PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 5/00 | A1

(11) International Publication Number: WO 95/12664

(43) International Publication Date: 11 May 1995 (11.05.95)

(21) International Application Number: PCT/US94/11535

(22) International Filing Date: 12 October 1994 (12.10.94)

(30) Priority Data:
08/146,860 3 November 1993 (03.11.93) US

(71) Applicant: GENETICS INSTITUTE, INC. [US/US]; 87 CambridgePark Drive, Cambridge, MA 02140 (US).

(72) Inventors: ADAMSON, S., Robert; 15 Cathy Road, Chelmsford, MA 01824 (US). DRAPEAU, Denis; 15 Norwood Road, Salem, NH 03079 (US). LUAN, Yen-Tung; 3 Armand Drive, Chelmsford, MA 01824 (US). MILLER, Douglas, Alan; 6 Alexander Avenue, Salem, NH 03079 (US).

(74) Agent: LAZAR, Steven, R.; Genetics Institute, Inc., 87 CambridgePark Drive, Cambridge, MA 02140 (US).

(81) Designated States: AU, CA, FI, JP, KP, KR, NO, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: ADAPTION OF MAMMALIAN CELL LINES TO HIGH CELL DENSITIES

(57) Abstract

Methods and nutrient media are disclosed useful for adapting mammalian cell lines to culture at increased cell densities.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

ADAPTION OF MAMMALIAN CELL LINES TO HIGH CELL DENSITIES

FIELD OF THE INVENTION

The present invention relates to improved methods of expressing proteins through culture of mammalian cell lines. In particular, the present invention relates to methods of improving the productivity of mammalian cell lines through adaption to otherwise growth-limiting conditions.

BACKGROUND OF THE INVENTION

It is known that various factors may be responsible for limiting the growth of cells at high cell densities. These factors include absence of sufficient amounts of nutrients needed by the cells for sustained growth, as well as the presence of growth-limiting concentrations of inhibitors that may be secreted by the cells in culture. One inhibitor that is secreted by mammalian cells is ammonia. See Miller et al., Bioprocess Engineering, 3:113-122 (1988); Inlow et al., United States Patent 5,156,964 describes a method for generating tolerance to ammonia that involves culturing cells in a medium to which ammonia has been added. Similarly, Schumpp et al., Cytotechnology, 8:39-44 (1992) describe a method for generating cell lines tolerant of both ammonia and lactic acid by culturing cells in a medium to which both ammonia and lactic acid had been added.

The previous methods have several drawbacks. First, in order to generate tolerance to an inhibitor according to the above methods, it is first necessary to determine that a particular inhibitor is a growth-limiting factor for cells and then to develop a protocol for generating tolerance to that inhibitor. Second, the growth of cell lines which are generated with tolerance to a particular inhibitor according to the above methods may then be limited by a second, different inhibitor. Repeated experiments may be necessary to generate tolerance to multiple growth-limiting inhibitors in order to achieve significant increases in cell densities.

SUMMARY OF THE INVENTION

According to the present invention, many of the drawbacks of the above prior art are overcome. The present invention provides methods by which the growth-limiting factors present for a particular cell line can be overcome without first conducting time-consuming testing to identify the specific growth-limiting inhibitors.

It is one object of the present invention to provide methods of improving the productivity of mammalian cell lines.

It is another object of the present invention to provide methods for adapting cell lines to high cell densities.

It is yet another object of the present invention to provide nutrient-rich growth media in which nutrients are present in sufficient quantity so that they are not expected to limit cell growth.

According to the present invention, the above objects are largely achieved by providing methods for adapting mammalian cell lines to culture at increased cell densities. The methods of the present invention comprise adapting mammalian cell lines to grow at increased cell densities, by (a) initiating a passage by diluting a culture containing mammalian cells with a suitable growth medium by a dilution factor suitable for the passage duration;
(b) maintaining pH, dissolved oxygen, and nutrients at non-limiting levels during the passage; and repeating steps (a) and (b) at least about 5 times. In a preferred embodiment of the invention, the steps are repeated about 5 to about 20 times.

The present invention further comprises methods for adapting CHO cell lines to grow to increased cell densities, comprising:

a) initiating a passage of duration aproximately 1 to 5 days by diluting a culture containing CHO cells at a density of at least approximately 1 x 10^6 cells/ml with a suitable growth medium, the dilution factor being suitable to the passage duration; (b) maintaining pH, dissolved oxygen, and nutrients in non-limiting levels during the passage; and

c) repeating steps (a) and (b) at least about 5 times.

The present invention further comprises methods for adapting CHO cell lines to grow to increased cell densities comprising: (a) initiating a passage of duration approximately 3 to 4 days by diluting a culture containing CHO cells at a density of at least approximately 1 x 10^6 cells/ml with a suitable growth medium, the dilution factor being suitable to the passage duration; (b)

maintaining pH, dissolved oxygen, and nutrients at non-limiting levels during the passage; and (c) repeating steps (a) and (b) at least about 5 times.

In a preferred embodiment, the present invention comprises a method for adapting mammalian cell lines to culture at increased cell densities, said method comprising continuously or periodically diluting a cell culture, containing mammalian cells, with a suitable growth medium, for between approximately 10 and 60 days, while maintaining pH, dissolved oxygen and nutrients at non-limiting levels.

Other preferred methods of the present invention comprise adapting CHO cell lines to culture at increased cell densities, said method comprising continuously or periodically diluting a culture containing CHO cells, at a density of at least approximately 1 x 10^6 cells/ml with a suitable growth medium, the dilution rate being less than approximately 0.029 hr^{-1}, while maintaining pH, dissolved oxygen and nutrients at non-limiting levels. Preferred dilution rates are between approximately 0.018hr^{-1} and 0.026hr^{-1}.

DETAILED DESCRIPTION OF THE INVENTION

Mammalian cell lines are used for the production of commercially useful proteins. Some mammalian cell lines which are commonly used include chinese hamster ovary (CHO) cell lines, hybridomas, monkey COS-1 cells, HeLa cells, melanoma cell lines such as the Bowes cell line, hybridoma cell lines, mouse L cells, mouse fibroblasts, mouse NIH 3T3 cells and the CV-1 cell line. In the present invention, these and other mammalian cell lines may be adapted for culture at high cell densities.

Suitable growth media for the present invention include any medium which provides nutrients at non-limiting levels. Nutrients will generally be at non-limiting levels if raising concentrations of all nutrients results in no increase in growth rate. Nutrient concentrations may be maintained at non-limiting levels by either providing excess amounts of all nutrients in the fresh medium or by adding nutrients to the culture as they are taken up by the cells or degraded. A suitable growth medium for mammalian cell lines is disclosed in Ling et al., Experimental Cell Research; 52:469-489 (1968). Accordingly, one preferred growth medium contains the amino acid nutrients in the concentrations disclosed in Table 1.

TABLE 1

Column I	Column II	Column III
NUTRIENT	CONCENTRATION RANGE (MG/L)	OPTIMAL CONCENTRATION (MG/L)
L-asparagine H_20	30-360	540
L-aspartic acid	69-798	266
Glycine	30-450	60
L-isoleucine	79-948	472
L-leucine	158-1890	681
L-lysine HCl	229-2742	728
L-methionine	75-894	238
L-serine	79-948	630
L-threonine	90-1074	381
L-tryptophan	31-366	131
L-tyrosine 2Na $2H_2O$	65-783	418
L-valine	141-1686	374

Other nutrients which may be addded to the medium include inorganic salts, such as chlorides, phosphates, sulfates and nitrates, sugars, vitamins, and additives such as glutamine, pyruvate, linoleic, thioctic, selenite, hydrocortisone, insulin.

Other preferred growth media suitable for mammalian cell lines include a medium containing the components described in Table 2 below.

TABLE 2

NUTRIENT COMPOSITION OF MEDIUM

Column I	Column II	Column III	Column IV
Components	Medium proposed by Ling et al. (mg/L) (1968)	Medium used for adaptation in Example (mg/L)	Preferred non-limiting medium (mg/L)
sodium chloride	7000	4600	4400
potassium chloride	375	624	310
calcium chloride, anhydrous	156	232	58
sodium phosphate, dibasic, anhydrous		142	
sodium phosphate, monobasic, hydrate		125	130
magnesium chloride, anhydrous		57	
magnesium sulfate, anhydrous	120	98	84
cupric sulfate, anhydrous	185	0.0016	0.0018
ferrous sulfate, anhydrous		0.68	0.91
ferric nitrate, nonahydrate	1.2	0.10	
zinc sulfate, septahydrate	0.86	0.86	0.92
sodium selenite		0.010	0.010
sodium bicarbonate		2440	2400
L-alanine	45-534	36	71
L-arginine	218-2616	600	760
L-asparagine hydrate	30-360	180	540
L-aspartic acid	67-798	133	270
L-cysteine hydrochloride hydrate		282	700
L-cystine dihydrochloride	23-281	125	
L-glutamic acid	103-1236	59	120
L-glutamine	212-2544	1168	1200

Components	Medium proposed by Ling et al. (mg/L) (1968)	Medium used for adaptation in Example (mg/L)	Preferred non-limiting medium (mg/L)
glycine	38-450	60	60
L-histidine hydrochloride hydrate	105-1260	126	290
L-isoleucine	79-948	210	470
L-leucine	158-1890	260	680
L-lysine hydrochloride	229-2742	291	730
L-methionine	75-894	104	240
L-phenylalanine	99-1188	165	330
L-proline	86-1032	138	280
L-serine	79-948	315	630
L-threonine	90-1074	190	380
L-tryptophan	31-366	33	130
L-tryosine disodium dihydrate	57-678	262	420
L-valine	141-1686	187	370
biotin	0.03	0.41	1.6
D-calcium pantothenate	5.0	4.5	18
choline chloride	350	18	72
folic acid	0.10	5.3	21
i-inositol	35	25	100
nicotinamide	20	4.0	16
pyridoxine hydrochloride		0.062	16
pyridoxal hydrochloride	2.5	4.0	
riboflavin	1.5	0.44	1.8
thiamine hydrochloride	1.0	4.3	18
vitamin B12	0.003	1.6	5.6

Components	Medium proposed by Ling et al. (mg/L) (1968)	Medium used for adaptation in Example (mg/L)	Preferred non-limiting medium (mg/L)
D-glucose	2000	6000	6200
sodium pyruvate		110	
linoleic acid	0.21	0.084	0.17
thioctic acid	0.70	0.21	0.42
putrescine dihydrochloride		2.2	2.0
polyvinyl alcohol		2400	2400
insulin or Nucellin	1.0	10	10
hydrocortisone		0.072	0.072
methotrexate		1.3	
soybean phospholipid		10	
fetal bovine serum		5000	
B-glycerophosphate, disodium	1000		
D-sorbitol	100		
oxalacetic acid	65		
thymidine	10		
deoxycytidine	11		
homocysteine thiolactate	8-90		
glutathione, reduced	31-372		
sodium molybdate, dihydrate	0.015		
vitamin A acetate	1.0		
vitamin D3	0.005		
a-tocopherol	7.0		
oleic acid	0.2		
arachidonate, methyl	0.02		

Components	Medium proposed by Ling et al. (mg/L) (1968)	Medium used for adaptation in Example (mg/L)	Preferred non-limiting medium (mg/L)
cholesterol	5		
ovo-lecithin	25		
ethanol	2000		

Suitable dilution factors (for passaging) and suitable dilution rates (for continuous culture) appropriate for adapting a particular mammalian cell line to grow to increased cell densities may be calculated using the formulas:

$$\text{dilution factor} = e^{(\mu t)}$$

$$\text{dilution rate} = \mu$$

where t is the duration in hours of the upcoming passage and μ is any quantity less than μ_{max}, preferably a quantity between approximately (0.6 x μ_{max}) and approximately (0.9 x μ_{max}). μ_{max} in hour^{-1}, is the specific growth rate of the cell line when none of the following extracellular factors limits growth: pH, dissolved oxygen, nutrient depletion and cell-generated inhibitors.

The magnitude of μ_{max} may be estimated without precise measurement in a variety of ways. For example, an estimate of μ_{max} may be generated as follows. First the maximum cell density attainable in a spinner flask using a common medium (such as a 1:1 mixture of DME and F12) is determined by suspending growth phase cells in this medium in the spinner flask and measuring the cell density on each subsequent day until cell density no longer rises. Next, growth phase cells are suspended in fresh medium in another spinner flask at a starting density approximately 10-fold below the maximum attainable density and cultured for approximately 2 days. This culture is diluted with fresh medium to the same starting cell density every two days for several passages. The estimate of μ_{max} is the growth rate observed during these passages, calculated using the following formula:

$$\mu_{max} = (\ln X_f - \ln X_i)/t$$

where X_f is the cell density at the end of a typical passage, X_i is the cell density at the beginning

of the same passage, and t is the duration of the passage in hours.

For CHO cell lines, a suitable dilution factor for a given duration of passage may be as follows: If the passage is approximately 1 day, a suitable dilution factor is less than about 2, preferably from about 1.5 to about 2. If the passage duration is approximately 2 days, a suitable dilution factor is less than about 4, preferably from about 2 to about 4. If the passage duration is approximately 3 days, a suitable dilution factor is less than about 8, preferably from about 3 to about 7. If the passage duration is approximately 4 days, suitable dilution factors are less than about 16, preferably from about 5 to about 13. If the passage duration is approximately 5 days, a suitable dilution factor is less than about 32, preferably from about 9 to about 23. For other mammalian cell lines, suitable dilution factors may be calculated on the basis of the maximum growth rate of the cell line. The maximum growth rate for a cell line may be determined as described above.

In the method of the present invention, relatively constant levels of pH, dissolved oxygen, and nutrients are maintained at non-limiting levels during the passage. This may preferably be accomplished by performing the adaption process in a bioreactor. pH may be maintained at the proper pH by addition of a suitable alkaline or acidic additive or buffer, for example sodium carbonate and sodium bicarbonate. Dissolved oxygen may be maintained by introduction of oxygen or air bubbles. If necessary, nutrient levels may be maintained by the addition of those nutrients which are depleted, or by addition of fresh growth medium.

In the present invention, mammalian cell lines, such as CHO cell lines, may be cultured at a suitable cell density, which may be approximately 1 x 10^6 cells/ml, in a suitable growth medium, and may be diluted in accordance with the above description.

The present invention is illustrated by the following examples. These examples do not limit the invention in any manner. It is contemplated that minor improvements and variations may be made which are part of the present invention.

EXAMPLES

The recombinant chinese hamster ovary cell (CHO) line E5F3G expresses recombinant human M-CSF, as described in Clark et al., United States Patents 4,868,119 and 4,879,227. As described below, the E5F3G cell line was adapted to grow to increased cell densities, and thereby generate higher concentrations of rhM-CSF.

E5F3G cells from a spinner flask were grown to a density of 1.24 x 10^6 cells/ml in approximately 1000 ml of a nutrient-rich medium (Table 2) in a 2-L bioreactor (passage 1 in Table 3).

These cells were then cultured for an additional ten 3-day or 4-day passages in the 2-L bioreactor (passages 2 through 11) in the nutrient-rich medium. During each passage, pH was maintained at between 7.0 and 7.2 by addition of sodium carbonate and sodium bicarbonate and dissolved oxygen was maintained at between 20% and 60% of air saturation by introduction of oxygen bubbles. Each 3-day passage was started by diluting the culture from the preceding passage by a factor between 5.1 and 6.3, while each 4-day passage was started by diluting the culture from the preceding passage by a factor between 6.0 and 14.3.

The beneficial effect on the cell line was evident during two subsequent passages (passages 12 and 13). For example, in passage 12, which was started at a density of 0.50 x 10^6 cells/ml, cell density reached 4.90 x 10^6 cells/ml, and rhM-CSF titer reached 32.6 ug/ml. In contrast, in passage 4, which had been started at a higher cell density (0.59 x 10^6 cells/ml), cell density had reached only 2.44 x 10^6 cells/ml and rhM-CSF titer had reached only 14.9 ug/ml.

Table 3: Adaptation of E5F3G cell line to increased cell densities

Passage number	Passage length (days)	Dilution ratio	Initial density (10^6/ml)	Final density (10^6/ml)	Final titer (ug/ml)
1	4	-	0.12	1.24	11.6
2	3	5.4	0.23	1.96	14.3
3	3	6.3	0.31	3.00	16.5
4	3	5.1	0.59	2.44	14.9
5	4	12.2	0.20	1.79	-
6	4	6.0	0.30	3.50	-
7	3	5.0	0.70	2.25	12.2
8	3	5.2	0.43	2.70	15.6
9	4	12.3	0.22	4.30	20.2
10	4	14.3	0.30	5.90	29.2
11	3	5.9	1.00	5.70	33.5
12	3	11.4	0.50	4.90	32.6
13	4	16.3	0.30	5.30	34.2

CLAIMS

We claim:

1. A method for adapting mammalian cell lines to grow at increased cell densities, said method comprising:

a) initiating a passage by diluting a culture containing mammalian cells with a suitable growth medium, the dilution factor being suitable for the passage duration;

b) maintaining pH, dissolved oxygen, and nutrients at non-limiting levels during the passage; and

c) repeating steps (a) and (b) at least about 5 times.

2. The method of claim 1, wherein steps (a) and (b) are repeated about 5 to about 20 times.

3. A method for adapting CHO cell lines to grow to increased cell densities, said method comprising:

a) initiating a passage of duration aproximately 1 to 5 days by diluting a culture containing CHO cells at a density of at least approximately 1 x 10^6 cells/ml with a suitable growth medium, the dilution factor being suitable for the passage duration;

b) maintaining pH, dissolved oxygen, and nutrients in non-limiting levels during the passage; and

c) repeating steps (a) and (b) at least about 5 times.

4. The method of claim 3, wherein steps (a) and (b) are repeated about 5 to about 20 times.

5. A method for adapting CHO cell lines to grow to increased cell densities, said method comprising:

a) initiating a passage of duration approximately 3 to 4 days by diluting a culture containing CHO cells at a density of at least approximately 1 x 10^6 cells/ml with a suitable growth medium, the dilution factor being suitable to the passage duration;

b) maintaining pH, dissolved oxygen, and nutrients at non-limiting levels during the

passage; and

c) repeating steps (a) and (b) at least about 5 times.

6. The method of claim 5, wherein steps (a) and (b) are repeated about 5 to about 20 times.

7. A method for adapting mammalian cell lines to culture at increased cell densities, said method comprising continuously or periodically diluting a cell culture, containing mammalian cells, with a suitable growth medium, for between approximately 10 and 60 days, while maintaining pH, dissolved oxygen and nutrients at non-limiting levels.

8. A method for adapting CHO cell lines to culture at increased cell densities,m said method comprising continuously or periodically diluting a culture containing CHO cells, at a density of at least approximately 1 x 10^6 cells/ml with a suitable growth medium, the dilution rate being less than approximately 0.029 hr^{-1}, while maintaining pH, dissolved oxygen and nutrients at non-limiting levels.

9. The method of claim 8, wherein the dilution rate is between approximately 0.018hr^{-1} and 0.026hr^{-1}.

10. A medium according to claim 10, wherein the nutrients are present in the following concentrations: L-asparagine H_2O, about 540 mg/l; L-aspartic acid, about 266 mg/l; glycine, about 60 mg/l; L-isoleucine, about 472 mg/l; L-leucine, about 681 mg/l; L-lysine HCl, about 728 mg/l; L-methionine, about 238 mg/l; L-serine, about 630 mg/l; L-threonine, about 381 mg/l; L-tryptophan, about 131 mg/l; L-tyrosine 2Na2H_2O, about 418 mg/l; L-valine, about 374 mg/l.

11. A medium suitable for the culture of mammalian cells at high cell densities, comprising components in the concentrations disclosed at columns III or IV of Table 2.

INTERNATIONAL SEARCH REPORT

Inter. nal Application No
PCT/US 94/11535

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US,A,3 850 748 (COOK, R.A.) 1974 see the whole document ---	1
X	GB,A,2 251 249 (MOGAM BIOTECHNOLOGY RESEARCH INSTITUTE) 1992 see page 8 ---	10
A	US,A,5 122 469 (MATHER, J.P.) 1992 see the whole document ---	1-11
A	US,A,5 156 964 (INLOW, D:) 1992 cited in the application see the whole document --- -/--	1-11

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 February 1995	22 -02- 1995

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Fernandez y Branas,F

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter. :al Application No
PCT/US 94/11535

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EXPERIMENTAL CELL RESEARCH, vol.52, 1968, NEW YORK pages 469 - 489 LING C.T. ET AL 'Chemically characterised concentrated corodies for comtinuous cell culture (the 7C'S culture media).' cited in the application see the whole document	1-11
A	R. IAN FRESHNEY 'Culture of Animal cells' 1987 , ALAN R. LISS, INC. , NEW YORK see page 127 - page 136	1-11

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter ial Application No
PCT/US 94/11535

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-3850748	26-11-74	NONE	
GB-A-2251249	01-07-92	FR-A- 2671098	03-07-92
US-A-5122469	16-06-92	NONE	
US-A-5156964	20-10-92	NONE	